Synthesis and Mode of Action of Oligomeric Sesquiterpene Lactones

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ARTICLE

Synthesis and Mode of Action of Oligomeric Sesquiterpene Lactones

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In this highlight we describe two case studies from our laboratory, involving the biomimetic syntheses and the biological mechanism elucidation of the bioactive oligomeric sesquiterpenoids, (+)-ainsliadimer A (**4**) and (–)-ainsliatrimer A (**5**). Ainsliadimer A possesses potent anti-inflammatory activity by inhibition of the NF-κB signalling pathway *via* binding at a previously untargeted allosteric site. (–)-Ainsliatrimer A induces apoptosis in cancer cells by activation of PPARγ. Furthermore, we highlight a new bioorthogonal ligation (TQ-ligation) developed in our laboratory which facilitates the target identification of complex natural products *via* pre-target fluorescence imaging and affinity chromatography. Generally, this paper will discuss the complete process from total synthesis to biological studies of complex natural products, and from the establishment of new bio-orthogonal chemistry to successful target identification. Our approach provides a systematic and efficient methodology for addressing the challenge of natural product target identification.

1 Introduction

Sesquiterpene lactones are a family of secondary metabolites derived almost exclusively from Asteracae/Compositae plants. Over 5000 members have been isolated,[1, 2] which display extensive structural diversity.[3] Biosynthesis of sesquiterpene lactones in plants is triggered by cellular stress, particularly as a response to microbial attack or oxidative damage, and also as anti-feedants to deter herbivores.[2, 4] Many sesquiterpene natural products display potent bioactivity in humans: (–)-parthenolide (**1**)[5] (**Figure 1**), the active component of the medicinal herb, feverfew, and (–)-helenalin (**2**)[6] from the *Arnica* genus have both demonstrated promising anti-tumour and anti-inflammatory properties. Parthenolide (**1**) is also the first small molecule known to selectively eradicate cancer stem cells in acute myeloid leukaemia.[7-9] In addition, (+)-artemisinin (**3**), isolated from the sweet wormwood plant and known in Chinese traditional medicine, is the predominant treatment for malaria in combination with several of its derivatives.[10] These discoveries have stimulated significant research interest into the synthesis and mechanism of action of sesquiterpene lactone natural products.

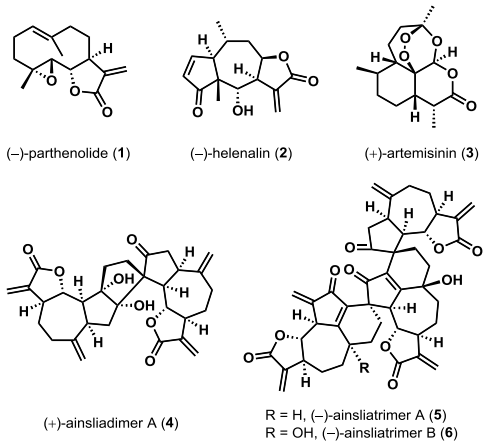

Figure 1. Chemical structures of selected sesquiterpene lactones.

Quantitative structure-activity relationship studies have highlighted several key structural features of sesquiterpene lactones which contribute to cytotoxicity in tumour and inflamed cells.[11-13] The presence of conjugated carbonyl groups, and particularly the α-methylene-γ-lactone ring, is essential for bioactivity of most compounds. It has been shown that sesquiterpene lactones *e.g.* **1** and **2**, selectively alkylate

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regulatory proteins *via* rapid Michael addition reactions with cysteine residues.[11] In addition, lipophilicity and conformational flexibility contribute to a lesser extent. Consequently, oligomers of sesquiterpene lactones, a natural product sub-class, which often possess a greater number of electrophilic centres and unusual molecular geometry are promising targets for biological evaluation as covalent drugs.

Sesquiterpene lactone oligomers are assembled biosynthetically by direct linkage of two or three monomeric sesquiterpene units, *via* cycloaddition or C-C coupling reactions. Numerous dimeric sesquiterpene lactones have been isolated (*i.e.* handelin, diguaiaperfolin, absinthin, inulanolides).[3] A notable example is ainsliadimer A (**4**), which retains potent anti-cancer and anti-inflammatory activity *vide infra.*[14] Trisesquiterpene lactones are much rarer, and to date only four have been isolated, including (-)-ainsliatrimers A (**5**) and B (**6**),[15, 16] anti-cancer compounds which will be discussed below.

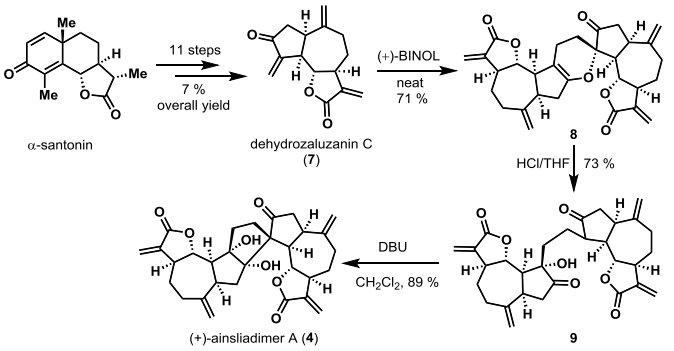
Since 2010, our laboratory has investigated the biological mechanism of action of several oligomeric sesquiterpenoids. Our dual objectives have been to assess the therapeutic potential of these compounds, and to study the biochemical pathways related to inflammation and cancer using chemical probe analogues. To these ends, the natural product scaffolds are modified in order to improve target binding affinity, generate analogues for SAR, and to enable conjugation of molecular tags, such as biotin or fluorophores which permit the purification or visualisation of protein targets, respectively. However, the selective chemical modification of natural products remains a significant challenge, due to their structural complexity and functional group diversity.[17, 18]

In this review, we describe two case studies involving the biomimetic syntheses and biological mechanism elucidation of (+)-ainsliadimer A and (–)-ainsliatrimer A, with a particular focus on chemical probe synthesis.

2. (+)-Ainsliadimer A (4)

2.1. Biomimetic Synthesis

(+)-Ainsliadimer A (**4**) was isolated by Zhang and co-workers from the whole plant extract of *Ainslia macrocephala*.[14] Initial biological studies suggested a potent anti-inflammatory activity, by inhibition of nitric oxide production in lipid polysaccharide (LPS) stimulated RAW264.7 cells. Its unprecedented structure, possessing a cyclopentane linkage between the monomeric units, was identified unambiguously by an X-ray crystal structure. Our group reported the first total synthesis of ainsliadimer A in 2010, using a biomimetic strategy.[19] The hetero-Diels-Alder precursor, dehydrozaluzanin C (**7**), was accessed in eleven steps from commercially available α-santonin (Scheme 1). The first step of this route involved a photocatalytic rearrangement to establish the required guaianolide [5-7-5] carbocyclic ring system,[20, 21] and then a sequence of dehydrogenative transformations were performed to introduce the carbon-carbon double bonds of dehydrozaluzanin C (**7**). With **7** in hand, several conditions were screened for the key hetero-Diels-Alder reaction. Activation of



Scheme 1: Synthesis of (+)-Ainsliadimer A (**4**)

the enone moiety using Lewis acids resulted in either substrate decomposition or poor yields. Fortunately, Brønsted acidic catalysts proved to be much more efficient, and (+/-)-BINOL effectively mediated the hetero-[4+2] cycloaddition to afford dimer **8** as a single diastereomer in 71% yield. Hydrolysis of **8** under mild acidic conditions yielded the α-hydroxy ketone **9** which readily underwent an intramolecular aldol condensation to furnish ainsliadimer A (**Scheme 1**). Dehydrozaluzanin C (**7**) also served as the key intermediate in our syntheses of other sesquiterpene oligomers, although other reaction conditions were required.[22] We next proceeded to investigate the biological activity of ainsliadimer A in greater detail.

2.2. Mechanism of Bioactivity

2.2.1. NF-κB Signalling Pathway

As discussed in section **1**, sesquiterpene lactones have emerged as promising anti-inflammatory and anti-cancer agents. Mechanistic studies on several monomeric sesquiterpenoids have shown that these compounds act upon the NF-κB signalling pathway, and we considered that ainsliadimer A may act *via* a similar mechanism.[22, 23] NF-κB is a transcription factor which is known to regulate the expression of over 150 genes related to immunitary response, cell stress, stimulation of cell proliferation and inhibition of apoptosis.[24] As such, high NF-κB activity required to coordinate inflammatory responses may result in the ability of neoplastic cells to resist apoptosis, providing a link between inflammation and cancer.[25, 26]

NF-κB is a homo- or heterodimer composed of combinations of five subunits: p50, p52, p65, RelB and c-Rel. The most commonly observed form is the p50:p65 heterodimer. However, all the subunits retain structural similarities including a DNA binding domain, a regulatory IκB binding domain and a conserved N-terminal sequence. NF-κB is usually retained in the cytoplasm in an inactive form by binding to IκB-α, a subunit of the inhibitory protein IκB. NF-κB activation and nuclear translocation are generally induced *via* one of two major pathways. In the canonical pathway, IκB-α is phosphorylated by IκB kinase (IKK) complex, leading to ubiquitination and proteasomal degradation.[27] The IKK complex is composed of three subunits: IKKα and IKKβ are kinases which catalyse phosphate transfer from ATP to IκB; while IKKγ/NEMO (NF-κB essential modulator) is a pseudo-kinase regulating the activity

of IKK. This pathway is induced by inflammatory stimuli, such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and LPS.

The non-canonical pathway shares several inducers with the canonical pathway, but in contrast does not entail IκB degradation.[28] Instead, the NF-κB inducing kinase (NIK) phosphorylates a NF-κB p100 subunit, leading to proteasomal processing into mature p52. Subsequent hetero-dimerisation with RelB induces nuclear translocation of the p52:RelB complex.

There are hundreds of known NF-κB inhibitors which act along both regulatory pathways (http://www.bu.edu/nf-kb).[29] A large majority target the kinase domain of IKKβ due its importance within the canonical pathway. However, to date there have been no clinically approved inhibitors, highlighting the need for novel molecules with unique binding properties and high selectivity which can act upon both pathways. Our investigations with ainsliadimer A, described in the following sections, revealed binding to a previously untargeted allosteric site within IKKβ.

2.2.2 Sesquiterpene Lactones and the NF-κB Pathway

The mechanism of action of few sesquiterpene lactones have been elucidated in detail. Parthenolide (**1**) is a potent inhibitor of NF-κB driven transcription.[5] Complete inhibition is observed at 5 μM concentration.[30] It forms a covalent bond with cysteine-38 of the NF-κB p65 subunit *via* a Michael addition reaction.[31] This was corroborated by introduction of cysteine-38 to serine point mutation which abolishes the effect of the small molecule even at 40 μM.[31] High concentrations of free cysteine abolish parthenolide activity, demonstrating the importance of the covalent bond.[31] Pull down experiments with a biotinylated parthenolide probe suggested that binding also takes place with cysteine-179 of IKKβ, although this latter interaction was only observed at high probe concentrations (30 μM).[32] Helenalin (**2**) acts *via* a similar mechanism, by modification of cysteine-38 in the NF-κB p65 subunit, although Merfort and co-workers have suggested that the α,β-unsaturated ketone of **2** may also alkylate a 2nd residue, cysteine-120 of p65.[22]

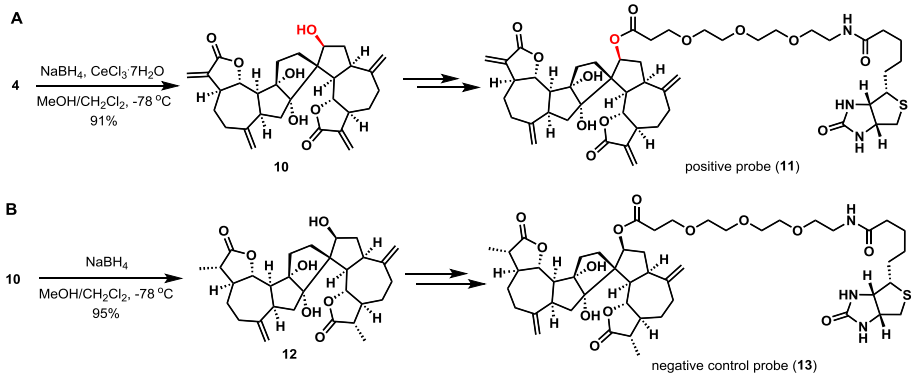
In order to identify the functional target(s) of ainsliadimer A, we prepared biotinylated probe analogues for affinity purification with streptavidin coated beads.[33] Waldmann *et al.* discuss the general workflow of this technique in an excellent recent review.[34] Usually, the biotin moiety is attached to the drug *via* a linker in order avoid steric hindrance between the matrix and target proteins. Many linkers are available, varying in hydrophilicity and conformational flexibility. In addition, it is essential to synthesise a negative control probe which is biologically inert although structurally similar to the active probe.[35] This method simplifies target identification by eliminating proteins which bind non-specifically with low affinity to the probe or linker. For covalent drugs, the specific residue modified may be determined by two complementary techniques: tandem mass spectrometry (MS/MS), and introduction of site selective point mutations. Once a putative protein target has been identified, further validation experiments are required to confirm binding between the molecule and protein of interest.[36]

2.2.3 Synthesis and Biological Evaluation of Ainsliadimer A Probes

Structural-activity relationships obtained during the total synthesis of (+)-ainsliadimer A indicated that ketone reduction at C3' and modification of the resulting secondary alcohol could be tolerated without loss of anti-inflammatory activity.[33] Consequently, the active probe (**11**) was synthesised by conjugation of a triethylene glycol linker to enhance water solubility, and biotin for affinity chromatography (**Scheme** 2). In expectation that the α-methylene-γ-lactone moieties play an essential role in the bioactivity of **4**, the conjugated olefins were selectively reduced in the negative control probe (**13**). Biological evaluation indicated that **11** effectively blocked NF-κB activation at 50 μM, whereas the negative probe **11** was completely inactive. Both probes were incubated individually with 293-T cell lysates, immunoprecipitated using streptavidin agarose beads and bound proteins were resolved using SDS-PAGE (**Figure 2A**). Two clear bands were precipitated specifically by the positive probe, which were identified as IKKα and IKKβ by trypsin digestion and fingerprint mass data analysis. This result was validated by equivalent experiments with purified IKKα and IKKβ proteins.

Amino acid sequence alignment of human IKKα and IKKβ indicated the presence of nine conserved cysteine residues which could potentially bind to the ainsliadimer A. Each of these residues were individually mutated to alanine, and the mutant proteins incubated with probe **11** (**Figure 2B**). Only the C46A mutants in both IKKα and IKKβ lost the ability to bind **11**, whereas the other mutants retained the binding affinity of the wild type. Tandem MS of a mixture of purified IKKβ with ainsliadimer **4** confirmed that only cysteine 46 was covalently modified.

Analysis of the crystal structure of IKKβ indicated that C46 is located in an allosteric site of the protein, and we verified that the C46S mutation did not influence kinase activity. However, molecular dynamics simulations showed that binding of ainsliadimer A does



Scheme 2. Syntheses of positive and negative control probes of (+)-Ainsliadimer A (**4**)

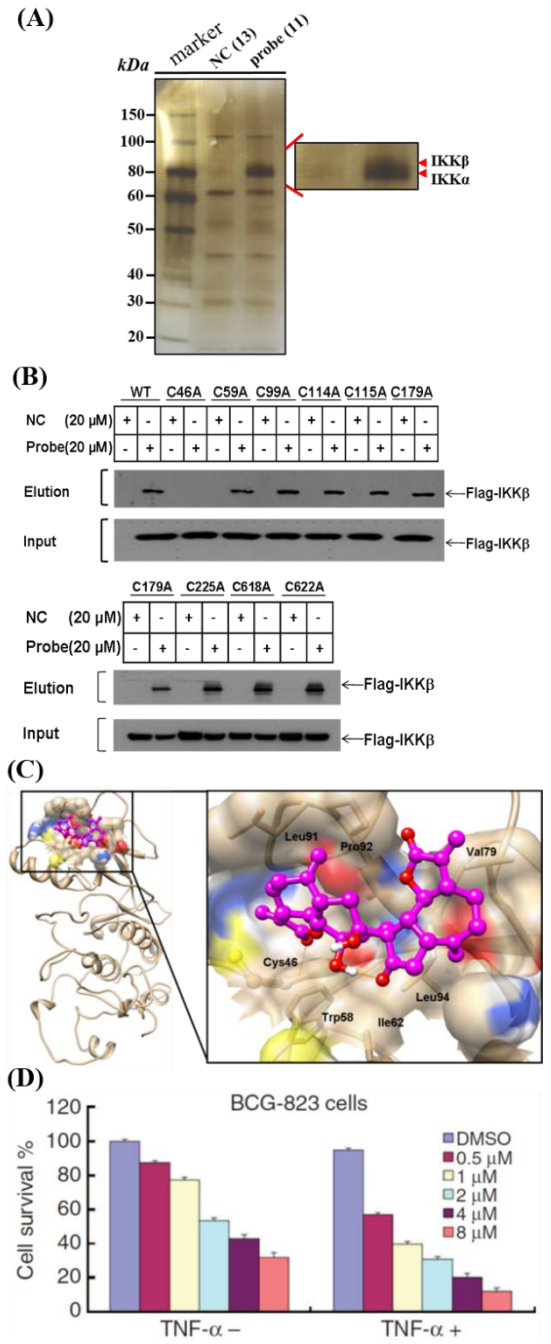


Figure 2: (A) SDS-PAGE and silver staining show that the positive probe **11** selectively binds to IKKα and IKKβ; (B) Nine IKK-β mutant proteins were generated by site-directed mutagenesis of cysteine for alanine residues. The C46A IKKα mutant lost binding ability to probe **13**, as shown by incubation with probes **11** and **13**, pull-down with streptavidin-agaraose, and resolution of precipitates by SDS-PAGE and biotin blotting; (C) A representative view of a simulated binding complex. A close up of ainsliadimer A (**4**) bound in an allosteric site, showing interactions with several hydrophobic residues; (D) Ainsliadimer A (**4**) shows synergistic anticancer activity against BCG-823 cells stimulated with TNF-α. The absence of TNF-α decreases cytotoxicity. Cell viability was determined by measuring ATP concentrations.

introduce significant conformational changes to the ATP binding site, thereby providing a mechanism for the small molecule induced loss of kinase activity, and subsequent inhibition of NF-κB activation. According to this computational study, several non-covalent interactions contribute to the binding affinity of ainsliadimer A to this allosteric site including a hydrogen bond between the backbone amide group of cysteine-46 and the carbonyl oxygen of the γ-lactone, as well as hydrophobic interactions with several non-polar residues; Trp58, Ile62, Val79 and Leu91 (**Figure 2C**). The close contact between ainsliadimer A and Trp58 was established experimentally by the tryptophan fluorescence quenching technique,[37] often used in the absence of a co-crystal structure. The binding affinity of 4 to IKKα/β, which is best expressed for covalent inhibitors as k_{inact}/K_i (the ratio of the rate constant for covalent bond formation (k_{inact}), to the dissociation constant for reversible binding (K_i)) was calculated experimentally to be 4.3 x 10^6 M^{-1} s^{-1}, showing fast kinetics for the forward Michael addition step (k_i = 7.7 min^{-1}) and strong non-covalent association (K_i = 30 nM). A systematic screening of the binding affinity of ainsliadimer A to 340 human kinases showed that 4 is selective for IKKβ and does not inhibit other enzymatic activity at 200 nM concentration.

In addition to its role in activating inflammatory responses, we observed that ainsliadimer A possesses micromolar cytotoxicity in BCG-823 gastric adenocarcinoma cells (**Figure 2D**). This property is synergistic with stimulation by TNF-α, and was shown to be due to the down-regulation of anti-apoptotic genes including c-FLIP, c-IAP and BCL-XL.

In summary, ainsliadimer A activates NF-κB *via* the canonical pathway. Although several inhibitors of IKKα/β have been reported, to the best of our knowledge ainsliadimer A is the first to bind specifically at cysteine-46, located in an allosteric site of the complex. We have speculated that due to its unique binding, ainsliadimer A or a derivative could be used as a probe for the elucidation of yet uncharacterised mechanisms of IKKα/β activation.[33] There is also significant potential for the development of **4** as a therapeutic candidate. Current studies are ongoing to improve its potency and pharmacokinetics (particularly water solubility) by SAR experiments and synthesis of pro-drugs.[8]

3 (–)-Ainsliatrimer A

3.1 Introduction

(–)-Ainsliatrimers A (**5**) and B (**6**) were isolated by Zhang and co-workers in 2008 from the extracts of the Chinese medicinal herb *Ainsliaea fulvoides*,[15] and initial biological evaluation demonstrated that these sesquiterpene lactones possess potent cytotoxicity against LOVO and CEM human cancer lines. Both compounds possess a highly complex undecacyclic scaffold and are distinguished only by C10-hydroxylation of ainsliatrimer B. Biosynthetically, it is proposed that three monomeric sesquiterpene lactone units are connected sequentially *via* [4+2]-cycloaddition reactions to form two intriguing spiro [4,5] decane linkages.[38]

We decided to investigate the functional protein target(s) of ainsliatrimer A (**5**), which showed the best anti-cancer relative to ainsliatrimer B and other derivatives.[39] We synthesised

positive and negative control probe analogues of ainsliatrimer A, modified with a bioorthogonal handle to allow conjugation of biotin and fluorophore reporter groups. Fluorescence tagging was also used to enable live-cell imaging and target localisation. The fluorophore was connected to ainsliatrimer A *in vivo* using TQ-ligation, a novel bioorthogonal reaction developed in our group and introduced in the following section. Overall, these experiments revealed that ainsliatrimer A inhibits the peroxisome proliferator activated receptor γ (PPARγ), a transcription factor involved in the induction of apoptosis and regulation of adipogenesis.[40]

3.2 TQ-Ligation

Bioorthogonal chemistry is a powerful tool for studying the function of biomolecules within the vast complexity of living cells. The reagents for bioorthogonal reactions must be inert to biochemical reactivity, and must fulfil several stringent requirements, including high reactivity and selectivity under physiological conditions (37 °C, pH 5-9), cell permeability, non-toxicity and product stability.[41, 42] Fast reaction kinetics are essential to ensure reaction completion at the μM or nM concentrations used to label biomolecules. Several bioorthogonal reactions have been reported within the past decade, notably tetrazine ligation[43] and strain promoted azide-alkyne cycloaddition (SPAAC),[44] and are used extensively to label proteins for fluorescence imaging or purification. Our group has developed the bioorthogonal TQ-ligation, a hetero-

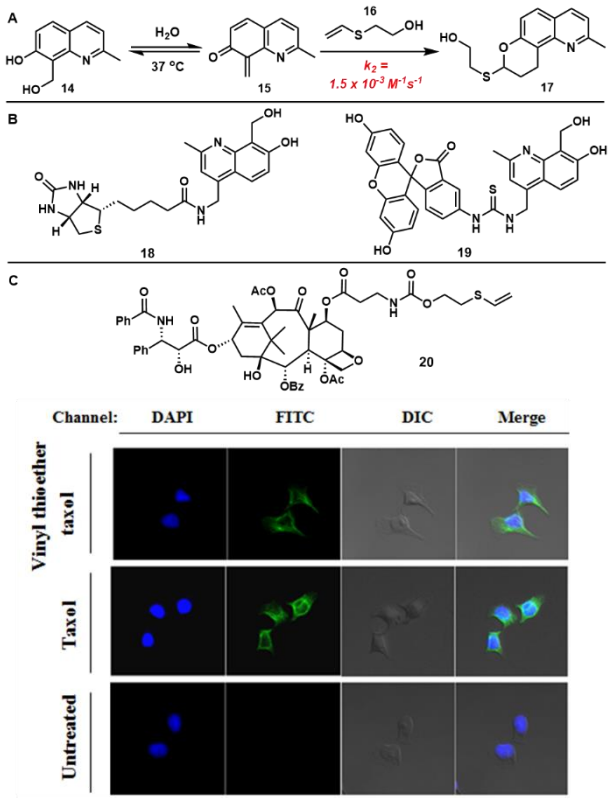
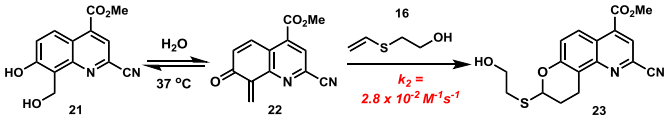


Figure 3: 1st generation TQ-ligation; (A) The original TQ-ligation between an *ortho*-quinoline quinone methide (*o*QQM) and a thio-vinyl ether (TV); (B) Structures of biotin- and fluorescein labelled *o*-QQM; (C) Imaging of TV-labelled taxol (**20**) in live HeLa cells.

Diels-Alder cycloaddition reaction between a thio-vinyl ether (**16**, TV) and an *ortho*-quinoline quinone methide (*o*QQM) (**15**), generated in situ under mild aqueous conditions from quinoline **14** (**Figure 3A**).[45, 46] The precursors and the thioacetal product are stable in the presence of oxidants and between pH 4-7.4. The *o*QQM reacts with cysteine residues *via* a Michael addition, but this reaction is reversible, whereas the cycloaddition with TV is irreversible and the desired cycloadduct is observed exclusively in competition experiments.[46] We synthesized both biotin (**18**) and fluorescein (**19**) labelled *o*QQM precursors for further applications in biological systems (**Figure 3B**). Both molecules are highly membrane permeable and non-cytotoxic to HeLa cells.[46] The utility of TQ-ligation has been demonstrated in the *in vivo* imaging of TV-labelled small molecule probes. Taxol was initially selected as a model system, as it is known to bind strongly to microtubules, a distinct intracellular structure for imaging. We synthesised the TV-labelled probe **20**, and incubated **20** with fluorescein-*o*QQM (**19**) within live HeLa cells. Confocal microscopy clearly showed specific staining of microtubule networks (**Figure 3C**), with a similar pattern to that obtained using standard immunostaining with an anti-tubulin antibody. Cells treated solely with **19** showed very low fluorescence, showing that background concentrations are insufficient for imaging.[47]

Recently, we have optimised the quinoline core to maximise reaction kinetics, and the 2nd generation TQ-ligation has a comparable 2nd order rate constant to the popular SPAAC reaction (**Scheme 3**).[48] For the biological evaluation of ainsliatrimer A we envisioned that TQ-ligation could provide a useful method to study its subcellular localisation in living cells, which would facilitate subsequent target identification.



Scheme 3: The 2nd generation TQ-ligation

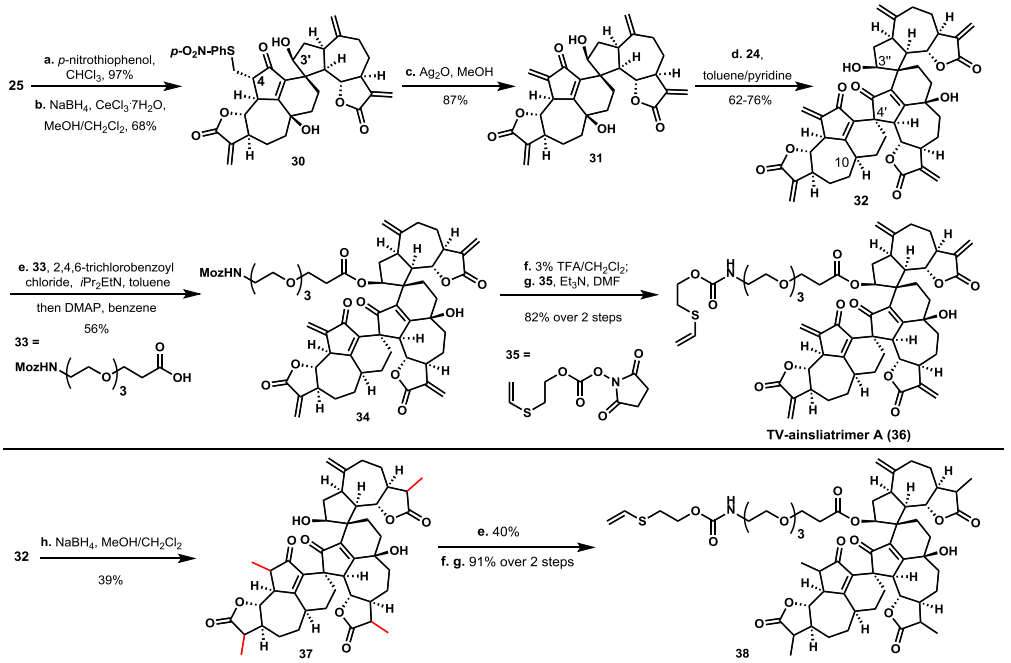
3.3 Synthesis of (–)-Ainsliatrimer A

Our group recently reported the first total synthesis of ainsliatrimers A and B.[49] This work relied heavily on insights obtained from previous syntheses of the dimeric sesquiterpene lactones, the gochnatiolides A-C,[50, 51] which appear to be biogenetic precursors of **5** and **6**. As shown in the retrosynthetic analysis (**Scheme 4A**), it was proposed that ainsliatrimer A could be accessed from diene **24** and gochnatiolide B (**25**) in a one pot cascade reaction. This would involve sequential [4+2]-cycloaddition and isomerisation of the C1-C10 double bond into conjugation with the C3 carbonyl. Ainsliatrimer B could be furnished by further radical allylic oxidation at C10.

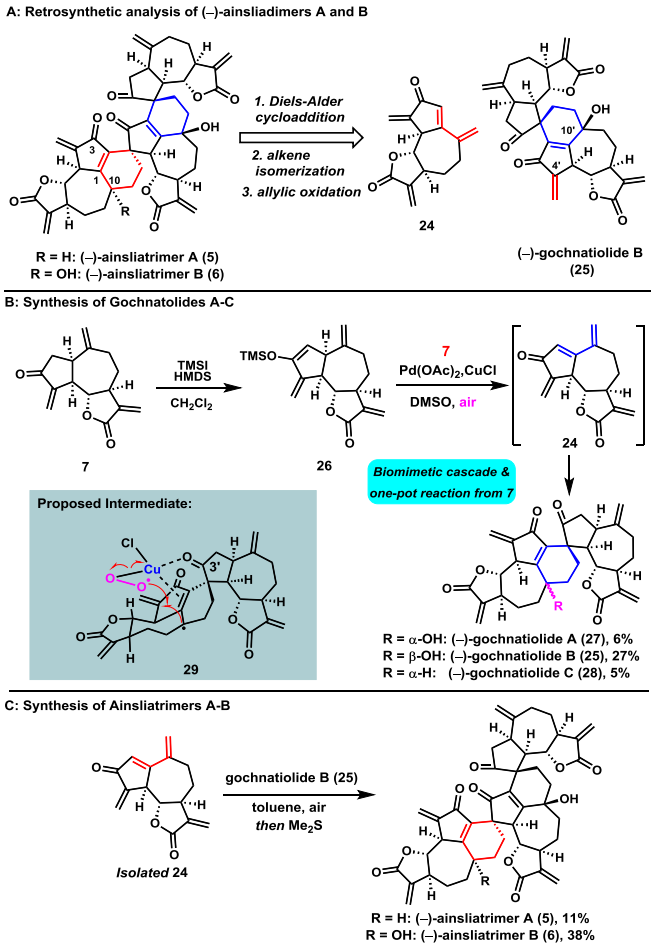
The gochnatiolides are ultimately derived from two molecules of dehydrozaluzanin C (**7**) which is also an important intermediate in the synthesis of ainsliadimer A (section 2.1). After extensive optimisation, we found that gochnatiolides A-C could be generated by Saegusa oxidation of TMS enol ether **26**,

intermolecular Diels-Alder cycloaddition, and radical initiated allylic oxidation (**Scheme 4B**). The product ratio for the latter step was controlled by the presence of reaction additives: in particular, CuCl increased selectivity for gochnatiolide B (**25**) by a chelate effect between a Cu(II) peroxo species generated from the reaction of CuCl with oxygen, and the C3' carbonyl oxygen (**29**). Under these conditions, **25** could be isolated in 27% yield, while gochnatiolides A (**27**) and C (**28**) were obtained in 6% and 5%, respectively. Comparison of the synthetic and natural NMR spectra of **25** resulted in the structural reassignment of the initially proposed structure.[52]

Scheme 4: Syntheses of (–)-ainsliatrimers A (**5**) and B (**6**).



Scheme 5. Syntheses of the chemical probe TV-ainsliatrimer A (**36**), and negative control probe **38**.



The final reaction cascade between gochnatolide B (**25**) and diene **24** was optimised by performing the reaction in toluene under high dilution, affording ainsliatrimers A (**5**) and B (**6**), in 11% and 38% yield, respectively (**Scheme 4C**).[49] The highlights of our synthesis include the unambiguous confirmation of the structures of complex natural products in the absence of an X-ray crystal structure, and the use of biomimetic cascade 'self-construction' reactions to rapidly build molecular complexity.

3.4 Synthesis and Biological Evaluation of Ainsliatrimer A Probes

In order to synthesise probe analogues of ainsliatrimer A (**5**), various strategies were employed to modify the natural product scaffold directly. However, the structural complexity of **5** prevented its chemo- and regio-selective functionalization. Instead, probes were synthesised by modification of the advanced intermediate, gochnatiolide B (**18**). This strategy, termed diverted total synthesis (DTS), is frequently used in the development of natural product based therapeutics.[53] Modifications of late-stage intermediates are used to explore the surrounding chemical space for structure-activity relationships, while simultaneously avoiding the synthetic challenges involved in natural product derivatisation.

We set out to synthesise an ainsliatrimer A analogue modified with the thio-vinyl ether (TV) moiety for conjugation to a fluorescent dye or biotin *via* TQ-ligation (**Scheme 5**).[39] The C3' position of gochnatiolide B (**25**) was suitable for modification, as analogues prepared for SAR retained the bioactivity of **25**. We observed that Luche reduction of the C3' carbonyl of gochnatiolide B (**25**) proceeded concomitantly with 1, 4-reduction of the *exo*-methylene at C4'. However, it was possible to protect this olefin as a Michael addition adduct with *p*-nitrophenol which could be subsequently removed by treatment with silver oxide in 87% yield. The desired trimeric structure of **32** was completed by the Diels-Alder cascade with diene **24** under anaerobic conditions. Direct esterification between alcohol **32** and a TV-modified linker was not successful under various conditions, but step-wise conjugation of a protected linker **33**, carbamate deprotection and N-hydroxysuccinimide mediated amide formation furnished TV-ainsliatrimer A **36** in 46% yield over three steps. The negative control probe was synthesised by treatment of trimeric **32** with excess $NaBH_4$, resulting global reduction of the *exo*-methylene groups.

The active probe **36** was initially used to explore the molecular localisation within living cells using TQ-ligation of a fluorescent tag. HeLa cells were incubated with ainsliatrimer A (**5**) or TV-labelled probe **36**, and both experiments were subsequently treated with the oQQM conjugated fluorescein **19**. Clearly stained, intracellular structures of the nucleus (**Figure 4**) were observed confirming that ainsliatrimer A accumulates in the nucleus and further suggesting that nuclear proteins or transcription factors are potential target(s). Subsequent target identification experiments were performed using affinity chromatography with the biotin labelled probes **39** and **40** (**Figure 5A**). On the assumption that some nuclear proteins may be unstable at 37 °C for the 12 h required for completion of the 1st generation TQ-ligation reaction, we decided to perform the TQ-ligation *in vitro* with **18** beforehand. Upon biotin probe incubation with the nuclear extracts of HeLa cells, precipitation with streptavidin-coated beads and SDS-PAGE, a clear band was specifically precipitated by the positive probe (**Figure 5B**). It was identified as histone deacetylase 2 (HDAC2) and peroxisome proliferator activated receptor γ (PPARγ) by trypsin digestion and fingerprint mass data analysis. However, gene knockdown experiments using siRNA suggested that HDAC2 is not a functional target. In contrast, the cytotoxicity of ainsliatrimer A, as measured by cell survival of HeLa cells was significantly reduced upon knockdown of PPARγ (**Figure 5C**) confirming that

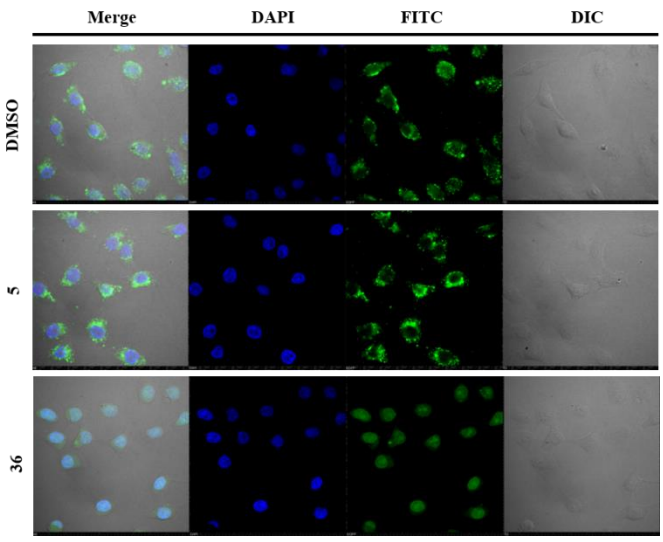


Figure 4: Pre-target imaging using (–)-ainsliatrimer A, positive probe **36** and *o*QQM-fluorescein **19**.

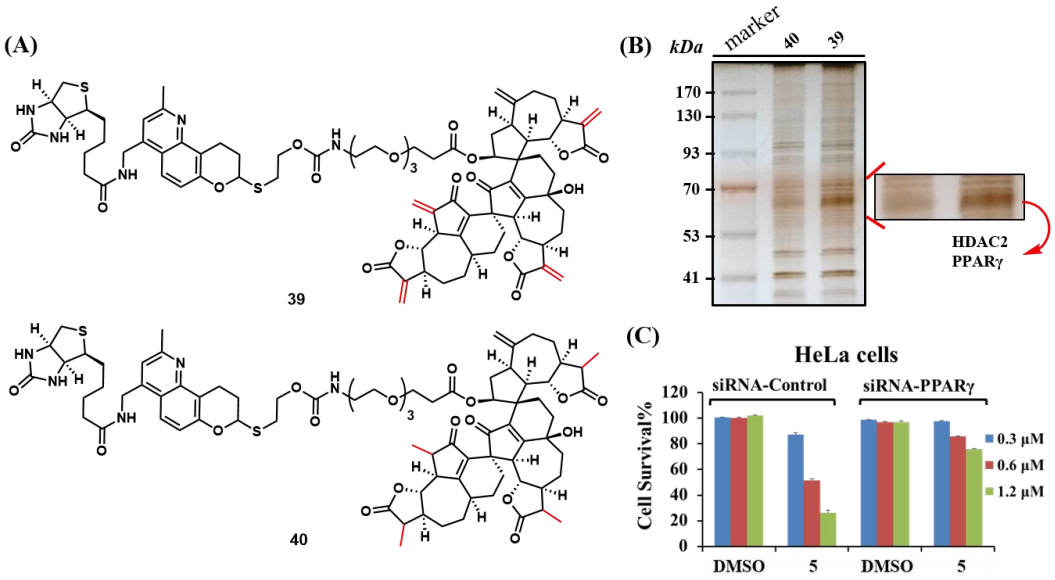


Figure 5. (A) Structures of positive and negative control probes of ainsliatrimer A (**39** and **40**). Probes were synthesised *in vitro* by TQ-ligation with biotin-*o*QQM (**18**); (B) SDS-PAGE and silver staining show that the positive probe **39** selectively pulls down two functional targets: HDAC2 and PPARγ; (C) Ainsliatrimer A loses cytotoxicity in HeLa cells with silenced PPARγ. expression.

ainsliatrimer A (**5**) binds specifically to PPARγ. We next investigated whether **5** acts as an agonist or antagonist of PPARγ. Co-incubation of HeLa cells with **5** and GW9662, a known PPARγ antagonist rescued the cell death induced by **5**, suggesting that **5** displays agonist activity. This was substantiated by the measurement of increased transcriptional activity of PPARγ and increased expression of COX-2, a known downstream target of PPARγ, which catalyses the synthesis of prostaglandin endoperoxide PGH2 from which many other prostaglandins are formed.[54] The prostaglandins are known ligands for receptor proteins (including PPARγ itself) which regulate cell proliferation, apoptosis and migration pathways. However, the direct link between PPARγ agonism and cancer is not well understood.[55] Interestingly, the sesquiterpene lactone natural product, deoxyelephantopin, also possesses anti-proliferative activity in HeLa cells *via* PPARg activation.[56] Full PPARγ agonists, such as the thiazolidinone class of anti-diabetics display cytotoxicity to several cancer cell lines *in vitro* and *in vivo*.[55] In combination, these experiments establish that **5** is an effective PPARγ agonist, although further studies are required to elucidate the precise mechanism of actions of this fascinating natural product.

4 Conclusion

Sesquiterpene lactones are a promising class of natural products, with applications as small molecule research tools in studies of protein function as well as potential therapeutics. They show high levels of selectivity for important cellular targets, despite possessing several electrophilic centres which covalently modify the protein target. Although traditionally viewed with caution, covalent modifiers are now receiving renewed interest:[57, 58] target identification is often facilitated as it is not necessary to introduce a cross-linking group to a probe before pull down experiments; and irreversible target modification increases the residence time within cells, an advantage in selective drugs.[59]

In this highlight, we have described the process of biological evaluation of two oligomeric sesquiterpene lactones, (+)-ainsliadimer A (**4**) and (–)-ainsliatrimer A (**5**). Ainsliadimer A inhibits the activation of NF-κB, a transcription factor known to induce expression of genes involved in inflammation, cell proliferation and apoptosis, and therefore a key protein in the connection between chronic inflammation and cancer. It directly inhibits the kinase activity of IKK *via* a novel mode of action by covalent bond formation with cysteine-46 located in an allosteric binding pocket. **4** is also cytotoxic towards gastric adenocarcinoma cells by induction of apoptosis. Ainsliatrimer A (**5**) possesses anti-cancer activity by associating to and increasing the activity of the transcription factor PPARγ, responsible for regulation of adipogenesis and the downstream biosynthesis of hormones such as the prostaglandins.

Due to the initial lack of natural material available by plant extraction, we completed concise biomimetic syntheses of ainsliadimer A (**4**), ainsliatrimer A (**5**) and the gochnatiolides A-C, which were formed in the synthetic pathway towards **5**, gaining sufficient quantities for full biological evaluation. Of particular note are the structural reassignment of gochnatiolide **B** (**25**), and the use of one-pot reaction cascades to establish the desired linkages between sesquiterpene lactone monomers in the gochnatiolides and **5**. Subsequently, the natural products were modified to enable conjugation of biotin for affinity purification, and a fluorophore for live-cell imaging. Ainsliatrimer A was connected to the fluorophore fluorescein *in vivo*, demonstrating the utility of TQ-ligation, a new

bioorthogonal reaction developed in our group involving the hetero-Diels-Alder cycloaddition between an *ortho*-quinoline quinone methide (*o*QQM) and a thio-vinyl ether. We have since reported an optimised 2nd generation TQ-ligation with reaction kinetics comparable to the widely used strain-promoted azide-alkyne cycloaddition reaction.

A recently published commentary by several leading chemical biologists describes the key properties of successful chemical probes.[35] These include high potency, well-characterised selectivity, a proven mechanism of action and the availability of appropriate control compounds. Our biological studies with both ainsliadimer A and ainsliatrimer A relied on readily accessible negative control analogues which could not form covalent bonds with protein targets but otherwise retained the natural product architectures. We were therefore able to confidently establish the high selectivity of both molecules, although further studies on the anti-cancer activity would be useful for ainsliadimer A, as these experiments were performed at high (μM) concentrations. Detailed studies are also required to determine the precise mechanism of action of ainsliatrimer A. Overall our work to date provides a model workflow for the transformation of natural products into efficient chemical probes and the identification of their protein targets. In the future, we hope that both **4** and **5** may be widely used as probes of their respective pathways, and as potential anti-inflammatory or anti-cancer therapeutics.

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6 References

1. M. Chadwick, H. Trewin, F. Gawthrop and C. Wagstaff, *International Journal of Molecular Sciences*, 2013, **14**, 12780-12805.
2. D. Chaturvedi, *ISBN: 978-81-308-0448-4, Research Signpost, Trivandrum*, 2011, 313-334.
3. Z.-J. Zhan, Y.-M. Ying, L.-F. Ma and W.-G. Shan, *Natural Product Reports*, 2011, **28**, 594-629.
4. J. K. Holopainen, *Trends in Plant Science*, 2004, **9**, 529-533.
5. A. Ghantous, A. Sinjab, Z. Herceg and N. Darwiche, *Drug Discovery Today*, 2013, **18**, 894-905.
6. G. Lyss, T. J. Schmidt, I. Merfort and H. L. Pahl, *Biological Chemistry*, 1997, **378**, 951-961.
7. M. L. Guzman, R. M. Rossi, L. Karnischky, X. J. Li, D. R. Peterson, D. S. Howard and C. T. Jordan, *Blood*, 2005, **105**, 4163-4169.
8. M. L. Guzman, R. M. Rossi, S. Neelakantan, X. Li, C. A. Corbett, D. C. Hassane, M. W. Becker, J. M. Bennett, E. Sullivan, J. L. Lachowicz, A. Vaughan, C. J. Sweeney, W. Matthews, M. Carroll, J. L. Liesveld, P. A. Crooks and C. T. Jordan, *Blood*, 2007, **110**, 4427-4435.
9. D. C. Hassane, M. L. Guzman, C. Corbett, X. Li, R. Abboud, F. Young, J. L. Liesveld, M. Carroll and C. T. Jordan, *Blood*, 2008, **111**, 5654-5662.
10. J.-M. Kindermans, J. Pilloy, P. Olliaro and M. Gomes, *Malaria Journal*, 2007, **6**.
11. B. Siedle, A. J. García-Piñeres, R. Murillo, J. Schulte-Mönting, V. Castro, P. Rüngeler, C. A. Klaas, F. B. Da Costa, W. Kisiel and I. Merfort, *Journal of Medicinal Chemistry*, 2004, **47**, 6042-6054.
12. A. Ghantous, H. Gali-Muhtasib, H. Vuorela, N. A. Saliba and N. Darwiche, *Drug Discovery Today*, 2010, **15**, 668-678.
13. A. C. Beekman, H. J. Woerdenbag, W. vanUden, N. Pras, A. W. T. Konings, H. V. Wikstrom and T. J. Schmidt, *Journal of Natural Products*, 1997, **60**, 252-257.
14. Z.-J. Wu, X.-K. Xu, Y.-H. Shen, J. Su, J.-M. Tian, S. Liang, H.-L. Li, R.-H. Liu and W.-D. Zhang, *Organic Letters*, 2008, **10**, 2397-2400.
15. Y. Wang, Y.-H. Shen, H.-Z. Jin, J.-J. Fu, X.-J. Hu, J.-J. Qin, J.-H. Liu, M. Chen, S.-K. Yan and W.-D. Zhang, *Organic Letters*, 2008, **10**, 5517-5520.
16. Y. Ye, X.-Q. Li and C.-P. Tang, *Chinese Journal of Natural Medicines*, 2010, **8**, 68-80.
17. O. Robles and D. Romo, *Natural Product Reports*, 2014, **31**, 318-334.
18. E. E. Carlson, *Acs Chemical Biology*, 2010, **5**, 639-653.
19. C. Li, X. Yu and X. Lei, *Organic Letters*, 2010, **12**, 4284-4287.
20. D. H. R. Barton, *Proceedings of the Chemical Society of London*, 1957, 205-205.
21. D. H. R. Barton, *Helvetica Chimica Acta*, 1959, **42**, 2604-2616.
22. G. Lyss, A. Knorre, T. J. Schmidt, H. L. Pahl and I. Merfort, *Journal of Biological Chemistry*, 1998, **273**, 33508-33516.
23. P. Rungeler, V. Castro, G. Mora, N. Goren, W. Vichnewski, H. L. Pahl, I. Merfort and T. J. Schmidt, *Bioorganic & Medicinal Chemistry*, 1999, **7**, 2343-2352.
24. M. S. Hayden and S. Ghosh, *Cell*, 2008, **132**, 344-362.
25. J. A. DiDonato, F. Mercurio and M. Karin, *Immunological Reviews*, 2012, **246**, 379-400.
26. M. Karin, *Nature*, 2006, **441**, 431-436.
27. H. Hacker and M. Karin, *Science's STKE : signal transduction knowledge environment*, 2006, **2006**, re13-re13.
28. S.-C. Sun, *Cell Research*, 2011, **21**, 71-85.
29. D.-F. Lee and M.-C. Hung, *Clinical Cancer Research*, 2008, **14**, 5656-5662.
30. P. M. Bork, M. L. Schmitz, M. Kuhnt, C. Escher and M. Heinrich, *Febs Letters*, 1997, **402**, 85-90.
31. A. J. Garcia-Pineres, V. Castro, G. Mora, T. J. Schmidt, E. Strunck, H. L. Pahl and I. Merfort, *Journal of Biological Chemistry*, 2001, **276**, 39713-39720.
32. B. H. B. Kwok, B. Koh, M. I. Ndubuisi, M. Elofsson and C. M. Crews, *Chemistry & Biology*, 2001, **8**, 759-766.

33. T. Dong, C. Li, X. Wang, L. Dian, X. Zhang, L. Li, S. Chen, R. Cao, L. Li, N. Huang, S. He and X. Lei, *Nature Communications*, 2015, **6**.
34. S. Ziegler, V. Pries, C. Hedberg and H. Waldmann, *Angewandte Chemie-International Edition*, 2013, **52**, 2744-2792.
35. C. H. Arrowsmith, J. E. Audia, C. Austin, J. Baell, J. Bennett, J. Blagg, C. Bountra, P. E. Brennan, P. J. Brown, M. E. Bunnage, C. Buser-Doepner, R. M. Campbell, A. J. Carter, P. Cohen, R. A. Copeland, B. Cravatt, J. L. Dahlin, D. Dhanak, A. M. Edwards, S. V. Frye, N. Gray, C. E. Grimshaw, D. Hepworth, T. Howe, K. V. M. Huber, J. Jin, S. Knapp, J. D. Kotz, R. G. Kruger, D. Lowe, M. M. Mader, B. Marsden, A. Mueller-Fahrnow, S. Muller, R. C. O'Hagan, J. P. Overington, D. R. Owen, S. H. Rosenberg, B. Roth, R. Ross, M. Schapira, S. L. Schreiber, B. Shoichet, M. Sundstrom, G. Superti-Furga, J. Taunton, L. Toledo-Sherman, C. Walpole, M. A. Walters, T. M. Willson, P. Workman, R. N. Young and W. J. Zuercher, *Nat Chem Biol*, 2015, **11**, 536-541.
36. C. J. O'Connor, H. S. G. Beckmann and D. R. Spring, *Chemical Society Reviews*, 2012, **41**, 4444-4456.
37. R. Murali, X. Cheng, A. Berezov, X. L. Du, A. Schon, E. Freire, X. W. Xu, Y. H. H. Chen and M. I. Greene, *Proceedings of the National Academy of Sciences of the United States of America*, 2005, **102**, 10970-10975.
38. C. Li and X. Lei, *Journal of Organic Chemistry*, 2014, **79**, 3289-3295.
39. C. Li, T. Dong, Q. Li and X. Lei, *Angewandte Chemie-International Edition*, 2014, **53**, 12111-12115.
40. M. Ahmadian, J. M. Suh, N. Hah, C. Liddle, A. R. Atkins, M. Downes and R. M. Evans, *Nature Medicine*, 2013, **19**, 557-566.
41. J. A. Prescher and C. R. Bertozzi, *Nature Chemical Biology*, 2005, **1**, 13-21.
42. E. M. Sletten and C. R. Bertozzi, *Angewandte Chemie-International Edition*, 2009, **48**, 6974-6998.
43. M. L. Blackman, M. Royzen and J. M. Fox, *Journal of the American Chemical Society*, 2008, **130**, 13518-+.
44. J. A. Codelli, J. M. Baskin, N. J. Agard and C. R. Berozzi, *Journal of the American Chemical Society*, 2008, **130**, 11486-11493.
45. Q. Li, T. Dong, X. Liu, X. Zhang, X. Yang and X. Lei, *Current Organic Chemistry*, 2014, **18**, 86-92.
46. Q. Li, T. Dong, X. Liu and X. Lei, *Journal of the American Chemical Society*, 2013, **135**, 4996-4999.
47. K. M. Stewart, K. L. Horton and S. O. Kelley, *Organic & Biomolecular Chemistry*, 2008, **6**, 2242-2255.
48. X. Zhang, T. Dong, Q. Li, X. Liu, L. Li, S. Chen and X. Lei, *ACS Chemical Biology*, 2015, **10**, 1676-1683.
49. C. Li, T. Dong, L. Dian, W. Zhang and X. Lei, *Chemical Science*, 2013, **4**, 1163-1167.
50. C. Li, L. Dian, W. Zhang and X. Lei, *Journal of the American Chemical Society*, 2012, **134**, 12414-12417.
51. D. Xia, Y. Du, Z. Yi, H. Song and Y. Qin, *Chemistry-a European Journal*, 2013, **19**, 4423-4427.
52. F. Bohlmann, M. Ahmed, J. Jakupovic, R. M. King and H. Robinson, *Phytochemistry*, 1983, **22**, 191-195.
53. R. M. Wilson and S. J. Danishefsky, *Journal of Organic Chemistry*, 2006, **71**, 8329-8351.
54. E. A. Meade, T. M. McIntyre, G. A. Zimmerman and S. M. Prescott, *Journal of Biological Chemistry*, 1999, **274**, 8328-8334.
55. P. Tontonoz and B. M. Spiegelman, in *Annual Review of Biochemistry*, 2008, vol. 77, pp. 289-312.
56. G. Zou, Z. Gao, J. Wang, Y. Zhang, H. Ding, J. Huang, L. Chen, Y. Guo, H. Jiang and X. Shen, *Biochemical Pharmacology*, 2008, **75**, 1381-1392.
57. R. Mah, J. R. Thomas and C. M. Shafer, *Bioorganic & Medicinal Chemistry Letters*, 2014, **24**, 33-39.
58. Q. Liu, Y. Sabnis, Z. Zhao, T. Zhang, Sara J. Buhrlage, Lyn H. Jones and Nathanael S. Gray, *Chemistry & Biology*, 2013, **20**, 146-159.
59. M. H. Potashman and M. E. Duggan, *Journal of Medicinal Chemistry*, 2009, **52**, 1231-1246.